Developmental Gene Expression in the Adhesive Toe Pads of *Anolis* Lizards

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Abstract

Anoles live in various levels of the tree canopy and have evolved unique adhesive toe pad morphologies that correlate with those differences in habitat (Losos & Ricklefs, 2009). Each toe pad is covered with setae, hair-like projections on the toe pad, that provide the toe pad its adhesive capabilities. Variations in these microscopic structures have been lesser studied than functional variation in the toe pad. Similarly, the specific genes responsible for development at the points of variation in time and location have also not been studied. The objective of my project was to identify the genes and location responsible for anatomical variation in toe pad structure of *Anolis sagrei*, a species with a relatively small toe pad, and *Anolis carolinensis*, a species with a relatively large toe pad. HCR in situ hybridization kits were used to make genes fluoresce so that expression could be visualized through microscope imaging. **Due to the COVID-19 response, data collection was unfinished and the data that I have collected is locked in the lab; however, I summarized the results I have found to date**. I am excited by one result in particular: the *FGFR2* gene gave indication of location being between the toe pad ridges.

Introduction

There are nearly 400 recognized species of anoles that reside in various levels of the tree canopy. The structural complexity at different levels of the canopy may impact the evolution of morphological features that account for mobility in how that environment is used. A key adaptation of anoles is their adhesive toe pads that aid in movement and survival in trees. These subdigital toe pads possess microscopic projections called setae. The adhesive mechanism essential for anoles perched higher in the canopy relies on the setae that make up the toe pad and the Van der Waals forces between them (Elstrott & Irschick, 2004).

Despite established macroscopic variation in toe pad morphology, such as toe pad size, the developmental bases of this variation has not been addressed. The ultimate origin of variation, in terms of the genetic and developmental bases, in both microscopic and macroscopic toe pad structure has yet to be studied between anole species. Genetic studies of general skin appendages across other species have been conducted, providing an idea of the specific genes involved in epidermal appendage development. In scales, avian feathers, and mammalian hairs, genes have been identified that play a role in morphogenesis and development of these appendages, such as *BMP* genes (Di-Poï & Milinkovitch, 2016). Although common genes involved in these processes have been identified, genes directly responsible for development at the point of variation between species of anoles have not been researched.

- The purpose of my research was to begin determining the genetic bases for evolutionary variation of *Anolis* toe pads in embryonic development
- I hypothesized that there will be at least one gene expressed late in anole embryotic development in the location directly underneath the toe pad ridges that represents the point variation between species arises.

Methods

I prepared *Anolis sagrei* and *Anolis carolinensis* embryos or hindlimb specimens at early and late embryo stages for imaging. HCR in situ hybridization kits were used to make genes of interest fluoresce at their location of expression using a 3-day protocol.

- Specimens dehydrated in 100% MeOH washes and stored at -20 ℃ with 2-4 specimens per test tube.

Day 1

- Rehydrated in 1 mL MeOH washes.
- Treated with Proteinase K in 1X PBST for 2-4 minutes (early stage embryos) or 10-15 minutes (late stage embryos).
- Added 4% PFA in 1X PBST for 20 minutes.
- 30% Probe Hybridization Buffer (PHB) warmed at 37 ℃ for 10 minutes.
- Washed twice in 1X PBST for 5 minutes.
- Prehybridized in 30% PHB for 3 hours at 37 ℃.
- Probe solution prepared in 30% PHB.
- From this point on, tubes were kept in the dark by foil due to probe light sensitivity. Tubes were always kept moving during washes to keep the solution mixing.
- Incubated in probe solution overnight at 37 ℃.

Day 2

- 30% Probe Wash Buffer (PWB) warmed at 37 ℃ for 10 minutes.
- Washed four times in 30% PWB for 15 minutes at 37 ℃.
- Washed three times in 5X SSCT for 5 minutes at room temperature.
- Pre-amplified in amplification buffer for 30 minutes at room temperature.
- Snap cooled hairpin storage buffer. Hairpins were heated for 90 seconds at 95 ℃ then cooled for 30 minutes at room temperature. Hairpins were then added to amplification buffer.
- Specimens bathed in the amplification solution overnight at room temperature.

Day 3

- Washed in series of 5X SSCT washes.
- 1X TrueBlack was added to the end of the protocol to minimize background fluorescence.

Imaging

- Specimens put into petri dish of 1% agarose gel and PBS.
- Microscope set to the red channel if 546-labeled hairpins were used or the green channel if 488-labeled hairpins were used.
- Fluorescence on entire embryos was looked for, then limbs of late-stage embryos were cut off and secured in the agarose.
- Images were taken only if fluorescence was present to indicate gene expression.
- Orientation was standardized so the pad was face up at a 45-degree angle. The middle pad for fingers and the longest pad for toes (the second digit) was imaged.
- Observations were recorded for back scales, hindlimb scales (left and right), hindlimb toe pads (left and right), front limb finger pads (left and right), and other for each gene, channel, and PK time.
- After imaging, all tubes were stored in 4 ℃.

Images were taken of successful fluorescence but could not be included due to campus closures.

References

Di-Poï, N., Milinkovitch, M. C. (2016). The anatomical placode in reptile scale morphogenesis indicates shared ancestry among skin appendages in amniotes. Science Advances,2(6). doi:10.1126/sciadv.1600708

Elstrott, J., & Irschick, D. J. (2004). Evolutionary correlations among morphology, habitat use and clinging performance in Caribbean Anolis lizards. Biological Journal of the Linnean Society,83(3), 389-398. doi:10.1111/j.1095-8312.2004.00402.x

Losos, J. B., & Ricklefs, R. E. (2009). Adaptation and diversification on islands. Nature, 457, Pp. 830-6

Results

Expression was recorded in terms of scales and digit pads. Qualitative observations up to the point of campus closures were as follows:

- ***SHH:*** no expression in early stage embryos; expression in finger pads of some late stage embryos.
- ***BMP2:*** no expression in early stage embryos; expression in finger and toe pads of some late stage embryos; nail expression observed.
- ***BMP4:*** no expression in late stage embryos.
- ***HOXB9:*** no expression in early stage embryos or late stage embryos; notochord expression observed.
- ***EDA:*** no expression in late stage embryos.
- ***COLII:*** no expression in late stage embryos; bone expression observed.
- ***CTNNB:*** expression in all finger pads and some toe pads of late stage embryos.
- ***FGF10:*** no expression in late stage embryos.
- ***FGFR2:*** expression in all toe pads of late stage embryos. Fluorescence was located between ridges, in the troughs, and surrounding the perimeter of each ridge.

Discussion

The results could not be discussed in depth or compared to previous research yet without study completion. Therefore, my prediction that there will be at least one gene expressed late in anole embryotic development in the site directly underneath the toe pad ridges that represents the point of variation between species could not be evaluated as supported or not just. However, *FGFR2* was a promising start since it provided a sense of location of expression. Rather than fluorescing inside, underneath, or on top of the toe pad ridges themselves, *FGFR2* was expressed in the troughs between ridges and surrounding the edges of each individual ridge. Further study with similar observations as this would indicate location is surrounding rather than my prediction of underneath the ridges.

Since this project was cut short, continuation in the future can begin with *FGFR2* by itself. The same protocol can be used to visualize *FGFR2* expression in both *A. sagrei* and *A. carolinensis* at several different embryo stages. This comparison would help pinpoint the timing of gene expression as well as location if consistent throughout several repetitions of the experiment. After *FGFR2*, other genes can become the point of focus, too. These studies require each gene to be observed in more embryo stages than just one as well as use of multiple anole species with established variation in their digit pads, such as the size between *A. sagrei* and *A. carolinensis*. Despite the sudden halt of research due to COVID-19 and unfinished findings, my study helped identify a gene, *FGFR2*, that should be studied more in these terms to help enhance understanding of the genetic bases driving developmental evolution in anole toe pad variation. Significant findings in this area could open up ideas for future genetic studies on developmental evolution of variation in other species and contexts.